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Perspective

The Impact of Alloantibodies on Clinical VCA Outcomes and the Need for Immune Tolerance

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Abstract: The functional outcomes and restoration of form after vascularized composite allotransplantation (VCA) have exceeded the results that could be achieved with current autologous surgical techniques. However, the longevity of VCA grafts has been limited due to the development of donor-specific antibodies (DSAs), and chronic rejection and graft failure occur despite long-term immunotherapy. Furthermore, despite widespread consensus that these non-life-saving transplants are beneficial for select patients, the application of VCA is limited by the need for lifelong immunosuppression. Therefore, attempts to achieve drug-free tolerance through safe and effective therapies are critical. This review highlights recent publications regarding alloantibody-mediated rejection (AMR) in various VCAs with a focus on the critical need for novel tolerance-inducing strategies. The development and implementation of effective methods of inducing tolerance, such as the use of anti-CD3 immunotoxins, could drastically improve VCA graft outcomes and recipient quality of life.

Keywords: alloantibody mediated rejection; vascularized composite allotransplantation; immune tolerance; humoral immune response; donor-specific antibodies

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1. Introduction

Vascularized composite allotransplantation (VCA) involves the transplantation of an allograft composed of heterogeneous tissue (muscles, skin, bones, vessels, and nerves), which serves as a single functional unit, for the repair of complex tissue defects. Some current VCA grafts that have been transplanted include the face, hands, abdominal wall, penis, and uterus [1]. In contrast to solid organ transplants, these grafts are not considered lifesaving but life-enhancing. This includes restoring form and functionality in patients with amputations, catastrophic injuries, and large tissue defects with no conventional reconstructive option. Since the development of novel immunosuppressive agents in the 1980s and 1990s, there have been a variety of successful VCAs [2], including over 40 face and 120 extremity transplantations performed worldwide [3]. However, with about 2 million people living with limb loss in the U.S. and 185,000 amputations performed annually, many more individuals could benefit from VCA [4]. This highlights the potential for VCA to serve as a therapeutic option for complex tissue injuries that are not sufficiently reconstructed by autologous tissue transfers.

The application of these transplants is limited by the development of acute and chronic rejection, as well as the need for lifelong multidrug immunosuppression [5]. The standard triple-drug regimen for immunosuppression is a combination of a calcineurin inhibitor, an antiproliferative agent, and a corticosteroid [6]. These medications, with different mechanisms of action, are commonly used in three phases: induction, maintenance, and treatment of rejection [7]. In the induction phase, high-dose immunosuppression is used immediately following transplantation, when the risk of rejection is greatest. Induction therapy can involve the use of monoclonal or polyclonal antibodies or higher doses of medications used for maintenance therapy [7]. Due to the intensity of this regimen, there

is an increased risk of post-transplant malignancies, infections, and organ dysfunction within recipients [8]. Off-target non-immune system drug toxicity can also occur, including but not limited to kidney toxicity secondary to high doses of calcineurin inhibitors [9]. Furthermore, there are concerns that due to the higher antigenicity of the skin, a main component of most VCA grafts, recipients require increased doses of immunosuppressive medications when compared to solid organ recipients [10,11]. Therefore, the requirement of lifelong high-dose immunotherapy and its potentially severe side effects decreases the risk-to-benefit ratio for VCA.

Effective treatment options for VCA graft recipients have led to excellent short-term graft survival. However, candidates for VCA are frequently sensitized, due to burns, blood transfusions, multi-parity, or prior organ transplantation, making them more susceptible to antibody-mediated rejection (AMR) [12]. Although primarily observed with discordant xenografts, allografts can be compromised within minutes to hours by the presence of preformed donor-specific antibodies (DSAs), leading to hyperacute AMR [13,14] (Figure 1). Acute AMR can also develop within days to months secondary to DSAs that bind to the graft endothelium and activate complement-dependent and independent mechanisms, leading to natural killer cell, polymorphonuclear neutrophil, and macrophage recruitment [15] (Figure 1). Interestingly, alloantibodies can develop de novo within months to years following VCA, and levels have been found to develop and continually increase, despite adequate immunosuppression [16]. Furthermore, DSA development can be triggered by inadequate or insufficient immunosuppression, leading to chronic AMR where activated effector cells and concomitant T-cell-mediated rejection compromise graft survival [17] (Figure 1).

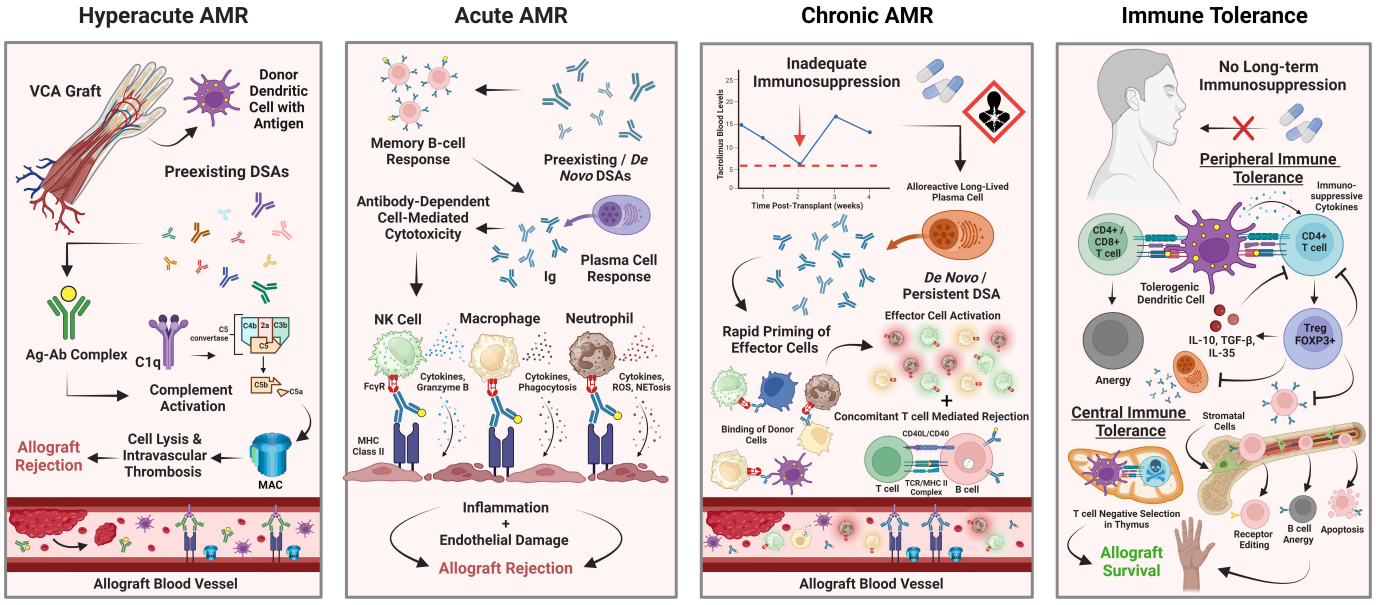

Figure 1. Cellular mechanisms of antibody-mediated rejection (AMR) and the need for immune tolerance. Hyperacute AMR occurs rapidly in the setting of preexisting donor-specific antibodies (DSAs). Antigen-antibody complexes cause acute graft injury by complement-dependent mechanisms. Acute AMR most often occurs secondary to preexisting DSAs. Antigen binding activates the memory B-cell response leading to plasma cell development and antibody production. Antibody-dependent cell-mediated cytotoxicity is carried out by natural killer (NK) cells which activate complement-mediated cell lysis and secrete various cytokines/chemokines that regulate immune responses. Chronic AMR can occur secondary to inadequate or inconsistent immunotherapy, resulting in de novo or persistent DSA production by plasma cells. A surge in DSAs causes the activation of effector cells that act in congruence with T cell-mediated rejection. Successful immune tolerance would eliminate the need for long-term immunosuppressive therapy. Peripheral immune tolerance utilizes tolerogenic cells that can suppress rather than activate the immune system. Central tolerance is achieved

through T cell negative selection in the thymus and/or B cell anergy, apoptosis, or receptor editing in the bone marrow. These strategies mitigate the immune response to donor tissue and lead to long-term allograft survival. VCA, vascularized composite allotransplantation; HLA, human leukocyte antigen; C, complement; Ag, antigen; Ab, antibody; MAC, membrane-attack complex; IgG, immunoglobulin G; IL, interleukin; FcγR, Fc-gamma receptors; MHC, major histocompatibility complex; TCR, T-cell receptor; TGF-β, Transforming growth factor beta; CD40L, CD40 ligand.

Considering the inability to prevent DSA development, induction of immunological tolerance would offer a way to avert AMR. Both central and peripheral tolerance mechanisms may co-exist to induce a state of immune unresponsiveness that is specific to donor antigens (Figure 1). Central tolerance includes the deletion or inactivation of alloreactive T cells once formed in the thymus [18]. In contrast, peripheral tolerance involves the deletion, inactivation, or regulation of reactive immune cells in the circulation [18]. These strategies largely focus on the cellular component of the immune response; however, many of the barriers to tolerance induction lie in the humoral immune response. This response includes B cells and their downstream production of cytokines and antibody-secreting plasma cells, which play a crucial role in both acute and chronic AMR [19] (Figure 1). Based on the significant clinical impact of DSAs on long-term graft survival [20–22], here, we provide an update on current evidence of AMR in the field of VCA, followed by the need for strategies to control the humoral alloimmune response and establish stable immune tolerance.

2. Methods

A PubMed, Embase, and Google Scholar search was conducted using the following search terms: "vascularized composite allotransplantation", "composite tissue allotransplantation", "alloantibody", "alloantibody mediated rejection", "donor-specific antibodies". Inclusion criteria consisted of articles, published since 2021, describing the presence of either pre- or post-operative DSAs in animal or human studies of VCA. Articles that focused on non-HLA antibodies, reviewed previously described cases, or had no mention of DSAs were excluded. In total, 6 articles describing evidence of DSA development in the setting of VCA were reviewed.

3. AMR in Animal VCA Models

VCA has been performed in a variety of animal models, including those that use non-human primates, swine, sheep, canines, rabbits, rats, and mice [23]. Antibody-mediated immune responses in experimental animal models closely resemble those of humans and have been shown to influence allograft survival. Recently, Shockcor et al. and Elgendy et al. evaluated the presence of AMR in non-human primate and swine VCA models, respectively. Shockcor et al. investigated a heterotopic hemifacial VCA modified by several different experimental models (Table 1) [24]. All animals received immunotherapy with consistent Tacrolimus and Mycophenolate Mofetil (MMF). In this study, Group 4 underwent heterotopic separation of the myocutaneous graft portion and the vascularized bone marrow (VBM) portion and demonstrated early Banff I-II rejection within 2 weeks of transplantation. Group 5, which underwent VCA with VBM transplantation after donor T cell depletion using antithymocyte globulin (ATGAM), demonstrated Banff IV rejection within 2 to 4 weeks of transplantation and developed both IgG and IgM DSAs, albeit at very low levels. These data suggest that heterotopic separation of the VCA graft and VBM as well as preoperative donor cellular depletion do not produce a survival advantage when considering postoperative DSA development and their effect on early rejection and graft loss.

Group 6 demonstrated no DSA development following irradiation of the donor graft and transplantation of the VCA graft with VBM components. At the time of necropsy, the VBM space was filled with viable CD3+ and CD19+ cells of recipient origin. On the other hand, Group 7, which experienced the same amount of donor graft radiation with bone marrow cell (BMC) infusion instead, demonstrated alloantibody formation in all recipients.

Furthermore, at the time of necropsy, the majority of the VBM space was necrotic. This finding confirms that the cell population within the VBM cannot be reconstituted using BMC infusion and alloantibody production, as well as their driven immune responses, are uncontrolled in the absence of VBM transplantation. Lastly, Group 8, which underwent VCA with VBM transplantation after recipient T cell depletion, developed IgG and IgM alloantibodies that increased an average of 2.7- and 18.3-fold, respectively. This finding endorses the idea that ATGAM T cell depletion leads to a homeostatic lymphopenia-induced proliferation of memory T cells and T regulatory cells, which then promote T follicular helper cell development and B cell activation and differentiation causing an increase in the production of high-affinity antibodies [25]. Furthermore, despite adequate T cell depletion and triple immunotherapy, acute rejection episodes occurred in the highly antigenic skin components of these allografts within days to weeks. The alloimmune responses observed in this study significantly interfered with early engraftment and overall graft survival, highlighting the substantial impact that DSAs can have on the overall success of VCA grafts.

Elgendy et al. investigated rejection outcomes of vertical rectus abdominus myocutaneous (VRAM) flap allotransplantation in SLA-mismatched miniature swine [26]. Immunotherapy included a combination of a co-stimulation blockade agent (cytotoxic T lymphocyte antigen 4-Ig (CTLA4-Ig)), and the mammalian target of rapamycin inhibitor (mTORi), rapamycin, with or without preceding calcineurin inhibition. There were two experimental groups (Table 1). Group A was treated daily with the mTORi and was given CTLA4-Ig (Belatacept) at various time points. Group B was treated daily with tacrolimus, followed by daily mTORi and CTLA4-Ig in the form of Belatacept (recipients B1 and B2) or Abatacept (recipient B3) at various time points. During the first 3 weeks post-transplantation, three out of five (60%) animals demonstrated increased levels of anti-donor antibodies. Despite continuous triple immunotherapy, animals B1 and B2 developed IgM alloantibodies. Similarly, in the setting of double immunotherapy, pig A1 developed IgG alloantibodies and experienced early grade I clinical rejection on POD 2, which quickly progressed to grade IV on POD 17. This rejection pattern correlates well with the almost doubling index of anti-donor IgG antibodies found in pig A1 during this period.

These results highlight the inability of combined immunotherapy to prevent early AMR in this VCA model. For animals B1 and B2, visual grade I rejection began to develop around POD 30. However, histological analysis revealed underlying grade I rejection by POD 14 and grade II by POD 21, with diffuse spongiosis and vacuolization of epidermal cells appearing by POD 29. Interestingly, in pigs B1 and B2, immunohistochemistry revealed IgG+ lymphocytes on POD 29 and 21, respectively. This demonstrates subclinical antibody-mediated histological changes within allografts subjected to extensive immunotherapy and highlights the potential for alloimmune responses to hinder VCA graft survival.

Table 1. Alloantibody development and study characteristics of animal VCA models.

Animal Model & Study	VCA Model	Immunosuppression	Experimental Groups	Sample Size (*n*=)	Alloantibody Development	Average Graft Survival (POD)
Non-Human Primates Shockcor et al. [24]	Heterotopic Hemifacial Allograft	Tacrolimus and Mycophenolate Mofetil	Group 1: VCA graft + VBM	4	No	348.3 ± 85.9
			Group 2: VCA graft	3	Yes	24.7 ± 16.6
			Group 3: VCA graft + BMC infusion	3	Yes	75.7 ± 44.1
			Group 4: Heterotopic myocutaneous graft + VBM	4	-	36.8 ± 20.7
			Group 5: VCA + VBM + donor T cell depletion with ATG	3	Yes	35.0 ± 26.2
			Group 6: VCA + VBM + 1.5 Gy donor irradiation	4	No	32.0 ± 21.4
			Group 7: VCA + 1.5 Gy donor irradiation + BMC infusion	3	Yes	20.7 ± 7.09
			Group 8: VCA + VBM + recipient T cell depletion with ATG + 21-day steroid taper	3	Yes	28.3 ± 8.02
Miniature Swine Elgendy et al. [26]	VRAM Flap Allograft	. CTLA4Ig (Belatacept) POD 0, 4, 7, 10, 14, then x1/week until POD 90 . Rapamycin POD −1–90	Pig A1	1	Yes	19.0 ± 2.83
			Pig A2	1	No	
		. CTLA4Ig (Belatacept) POD 7, 14, 21, 28 . Rapamycin POD 7–90 . Tacrolimus POD 0–13	Pig B1	1	Yes	71.5 ± 3.54
			Pig B2	1	Yes	
		. CTLA4Ig (Abatacept) POD 7, 14, 21, 28 . Rapamycin POD 7–90 . Tacrolimus POD 0–13	Pig B3	1	No	58.0

VCA, vascularized composite allotransplant; VRAM, Vertical rectus abdominus myocutaneous; VBM, vascularized bone marrow; ATG, anti-thymocyte globulin; BMC, bone marrow cells; MFI, mean fluorescence intensity; CTLA4Ig, cytotoxic T lymphocyte antigen 4-Ig.

4. AMR in Human Facial VCAs

Facial VCA can restore the appearance, facial functioning, and psychosocial well-being of severely injured patients [27]. However, despite these benefits, its widespread use is hindered by the risk of allograft rejection. Unlike rejection in other VCA grafts, complete loss of facial allografts can be life-threatening, and these patients do not have the option to return to a pretransplant state [28]. This highlights the importance of understanding short- and long-term AMR patterns in facial VCA grafts so that early diagnosis and management can be implemented. For this purpose, Moktefi et al. completed a long-term follow-up of six face transplant recipients, focusing on the histological presence of chronic and mucosal rejection [29]. Three of the patients (50%) had pre-formed anti-HLA antibodies (4, 6, and 7), while two (33.3%) developed de novo DSAs (4 and 5) (Table 2). Interestingly, four of these patients also developed lichen planus-like chronic rejection over the course of 52 ± 17 months post-transplantation. For patient #4, de novo class I and II DSAs were detected before any clinical signs of chronic rejection, at 44 and 11 months post-facial VCA, respectively. In contrast, patient #5 developed DSAs at 80 months, after the development of scleroderma-like chronic rejection, due to a reduction in immunosuppression following a tacrolimus-related renal injury. However, despite a return to adequate levels of maintenance therapy (methylprednisolone, MMF, and tacrolimus), the combination of DSAs and vasculopathy with $C4d^+$ wall deposits led to graft loss in this patient. Of note, the graft loss occurred nine months following de novo DSA development. This highlights the significant graft damage that AMR-associated vasculopathy can cause and the enduring risk of graft loss in VCA patients regardless of adequate immunosuppression.

Depending on the patient's needs, various types of facial allografts can be used. These include soft tissue only, soft tissue and bone, partial face, or full-face grafts [30]. Considering the heterogeneity of these grafts and the small population that receives them, it is important to understand the general outcomes of facial VCA grafts, including the overall risk for AMR. Kiukas et al. recently evaluated the four- and six-year outcomes of two patients following facial VCA (Table 2) [31]. Patient #1 was evaluated 6 years after receiving a partial facial allograft. Despite preformed DSAs and the development of additional DSAs within the first-year post-transplant, at follow-up, patient #1's HLA antibody levels were below preoperative levels and DSA levels were undetectable. In addition, thus far, there have been no clinical or histological signs of rejection in their allograft. Patient #2 was evaluated at 4 years post-transplant and received a full-face allograft. Interestingly, with an HLA mismatch of 4/2, this patient did not develop DSAs or exhibit clinical/histological signs of rejection at follow-up. The authors attribute the successful immunological outcomes and lack of rejection episodes in these patients to a "very strict patient selection of fully compliant patients", close follow-up, and prompt management of any issues.

Kiukas et al.'s follow-up protocol includes weekly appointments and punch biopsies for the first 3 months, which are reduced to every 6 months after 2 years. In addition, antibodies are measured every 6 months for two years and then yearly thereafter. This highlights the extensive patient contact needed to monitor DSA levels in the postoperative period and manage early developments of rejection. Although effective in this study, such a rigorous follow-up schedule may not be feasible for all the patients who could benefit from facial VCA. Studies have found that patients with higher degrees of social complexity (underrepresented minority status, limited English proficiency, and use of public health insurance) and mental illness demonstrate increased "lost-to-follow-up rates" [32]. Unfortunately, persons from racial-ethnic minority groups also have disproportionately poor mental health [33] and are often viewed as less compliant by physicians [34]. Furthermore, out of the 48 recorded facial VCAs, 21 (43.7%) have been performed for ballistic facial traumas, with several injuries described as self-inflicted [35,36]. This indicates the potential presence of undertreated mental health conditions within this population of need. Therefore, there are ethical concerns regarding implementing a stringent patient selection and follow-up process for individuals who may not be able to reach such standards due to systemic barriers. Instead, efforts should be aimed at inducing tolerance in VCA recipients,

which would allow for more patients to benefit from this procedure and decrease racial and socioeconomic disparities within the field. Furthermore, VCA recipients' quality of life could improve drastically as a state of immune tolerance would require less frequent appointments and invasive procedures.

Table 2. Alloantibody development in facial VCA studies, the time course for chronic rejection (CR) development, and histological follow-up.

Study	Patient #	Facial VCA Model	Alloantibody Development	Time from VCA to CR (Months)	Max Histological Follow-Up (Months)
Moktefi et al. [29]	1	Partial	No	35	153
	2	Full	No	-	122
	4	Full	Yes	77	124
	5	Full	Yes	48	89 **
	6	Full	No *	48	94
	7	Full	No *	-	40
Kiukas et al. [31]	1	Partial	Yes	-	72
	2	Full	No	-	48

CR, Chronic rejection; HLA, Human leukocyte antigen. * Presence of pre-transplant anti-HLA antibodies. ** Patient experienced graft loss at this time point.

5. AMR in Human Upper Extremity VCAs

Upper extremity VCA grafts, much like facial allografts, restore body wholeness through the replacement of tissue that is both visible and touchable. The surgical procedures involved in hand VCA are lengthy and technically demanding; however, managing the potential postoperative immune responses to the allograft is far more complex. In addition, the consequences of DSAs in solid organ recipients are well known [37–39]; however, due to a lack of large-scale trials of immunosuppression in VCA, the clinical significance of DSAs in upper extremity transplantation remains unestablished. Azoury et al. recently described a successful bilateral hand transplant in a 40-year-old woman highly sensitized to class II HLA antigens (Table 3) [40]. The donor was selected based on HLA class II matching and the absence of DSAs in the recipient's sera. Ultimately, the patient had one DRB1 mismatch (DR15) and one DRB5 mismatch (DR51). She was conditioned using five doses of thymoglobulin and received maintenance therapy in the form of Tacrolimus, mTORi, MMF, and prednisone. Tissue biopsies carried out for 1-year post-transplant indicated only transient Banff grade 1 acute rejection on POD8 and grade 2 at one-month post-transplant. Interestingly, on POD8, DSAs against DR51 and DR15 rose to weak-moderate levels (peak MFI levels 4938 and 2414, respectively) and did not subside until 8 months post-transplant. This case highlights the importance of donor compatibility and the potential for DSA development in the setting of consistent immunotherapy. Of note, a contributing factor in the authors' decision to select a donor based on HLA class II matching and the absence of DSAs was the high likelihood that this patient would need a future kidney transplant. This was predicted due to the patient's history of acute renal failure and likely renal toxicity secondary to the long-term use of Tacrolimus. Azoury et al.'s actions emphasize the challenges of selecting optimal VCA donors and encourage debate around the heavy risks of long-term immunotherapy, which may outweigh the benefits of non-lifesaving VCA grafts.

As previously illustrated, graft vasculopathy is one of the most severe features of chronic rejection and can quickly result in VCA graft loss. Vasculopathy causes ischemic-necrotic damage through myointimal proliferation and subsequent narrowing and/or obstruction of graft arteries [41]. DSAs can activate complement deposition in the endothelium via the classical pathway and bind to endothelial cells, causing vasculopathy in a complement-independent manner [42]. Considering this, DSA monitoring as well as other

techniques of assessing early signs of vasculopathy can allow for early detection of this destructive complication. Petruzzo et al. were able to use high-resolution ultrasonography to assess intimal media thickness (IMT) of radial and ulnar arteries in seven bilateral upper extremity transplant (UET) patients [43]. IMT was monitored annually for each patient, with follow-up periods ranging from 6 months to 13 years. After comparing the UET patient's results to seven matched healthy subjects, there was no statistically significant difference in IMT found. However, in patient #2, there was a significant difference between the IMT of the patient's native radial and ulnar arteries and those of the UE allograft ($p = 0.005$ and $p = 0.003$, respectively). This finding was present at all follow-up points over 6 years. Of note, this patient experienced a total of four acute rejection episodes, two during the first post-transplant year and two in the tenth. De novo DSAs were found during the seventh-year post-transplant (class II anti-HLA antibodies, MFI 1900) (Table 3) and waxed and waned for two years until the patient developed clinical signs of chronic rejection and histological evidence of vasculopathy, necessitating the removal of the allograft. Although patient #2's UE allograft demonstrated continuously thicker arteries, without baseline IMT measurements, this finding can neither be connected nor separated from their development of de novo DSAs. Interestingly, patient #5 also developed DSAs; however, there was no significant difference in their native vs. graft IMT measurements. This study highlights the perpetual risk of de novo DSA development following VCA and its potential influence on graft vasculopathy and loss in immunotherapy-compliant patients. Inducing tolerance in this patient population could limit these significant life-long risks, allowing for hasty graft accommodation and expansion of clinical VCA application.

Table 3. Alloantibody development, timing of DSA formation, and maximum length of follow-up in human upper extremity VCA models.

Study	Patient #	Alloantibody Development	Time from VCA to DSA Development (Months)	Maximum Follow-Up (Months)
Azoury et al. [40]	1	Yes	26	12
Petruzzo et al. [43]	1	No	-	156
	2	Yes	84	108
	3	No	-	60
	4	No	-	60
	5	Yes	60	36
	6	No	-	6
	7	No	-	6

6. Therapies for AMR in the Setting of VCA

There are currently no FDA-approved treatments for AMR after VCA and only a handful of randomized controlled trials have been conducted to date. The standard-of-care treatment is generally plasmapheresis with either low- or high-dose intravenous immunoglobulin (IVIG) or immunoadsorption (immunoglobulin selective apheresis). However, studies have found that antibody removal approaches are more effective when used in combination with treatments targeting the rejection process [44,45]. These adjunct strategies include rituximab, alemtuzumab, basiliximab, proteasome inhibitors (bortezomib), complement inhibitors, and T cell-depleting agents [46–48]. Typically, these treatments are used in combination and studied in the absence of a control group. Therefore, it is difficult to interpret the therapeutic potential of each independent modality.

Rituximab, a monoclonal antibody against the CD20 antigen, blocks B cell activation and maturation to antibody-producing plasma cells, although it does not affect existing plasma cells [49]. When used in combination with IVIG it is more effective in

preventing AMR episodes, allograft glomerulopathy, and DSA rebound formation [50]. Rituximab therapy has been shown to successfully reverse AMR with complete remission of clinical symptoms in a case of bilateral forearm transplantation where the patient developed AMR nine years post-transplant [47]. Alemtuzumab has been used by several groups as induction therapy [51,52], either as monotherapy [52] or triple therapy including tacrolimus, MMF, and steroids [51,53]. In solid organ transplant recipients, Alemtuzumab has shown the lowest rates of rejection and the highest success in eliminating the need for steroids when compared to other induction therapies [54]. However, long-term follow-up data assessing the prevention of AMR using alemtuzumab are scarce. Proteosome inhibitors, such as bortezomib, target plasma cells that produce DSAs and have shown some success in treating AMR. In a multicenter study treating 60 patients with two cycles of bortezomib, rituximab, and plasmapheresis, nearly 50% of patients achieved a >50% reduction in DSA levels [55].

Furthermore, although data are limited, complement inhibitors such as eculizumab (anti-C5 monoclonal antibody) are employed for cases of refractory AMR. In the setting of severe AMR episodes, eculizumab has been found to be more effective at preventing allograft loss when used in combination with splenectomy [56], or when used promptly [57]. Additionally, methods to moderate AMR include the inhibition of T cells, which leads to a decrease in T cell-dependent B cell responses. Inhibitors of T cell division (mycophenolate mofetil and steroids), and IL-2 signaling to T cells (calcineurin inhibitors), as well as T cell-depleting agents (antithymocyte globulin), can be added to treatment plans for chronic AMR when there is a concern for concomitant T cell-mediated rejection [58].

All in all, there is a need for a more effective treatment option for AMR following VCA, one that reduces inflammation and prevents further antibody formation without altering normal immune responses. However, the development of stable immune tolerance would eliminate the long-term risk of AMR and the need for such therapies.

7. The Need for Immune Tolerance Strategies in VCA

The risk-to-benefit ratio for VCA remains intensively debated. Although these patients do not have life-threatening medical conditions and are relatively "healthy" before transplantation, VCA can provide invaluable mental and physical health benefits, resulting in a greatly improved quality of life. However, nonspecific immunosuppression is currently the only established method for sustaining these allografts. Tissues, such as the skin, are highly antigenic, and thus VCA graft recipients must not only be on chronic immunosuppression, but these therapies must be maintained at a high level to prevent graft loss [59]. This higher-level drug regimen has been successful at preventing early graft failure, but it is insufficient in preventing both episodes of acute rejection of the skin and chronic rejection [19,60]. When the collective risk of complications is considered, lifelong immunosuppression can be thought of as a chronic disease that contributes to the morbidity burden of VCA patients. Prolonged immunosuppression increases the risk of infection, fracture, neoplasia, drug toxicity, and metabolic derangement [61]. These significant complications diminish the quality-of-life benefits that are gained from a successful VCA graft. Therefore, stable donor-specific allograft tolerance induction is needed to unchain transplant patients from life-long immunosuppressive therapy and expand this "life-restoring" treatment option to many more patients in need.

Transplantation tolerance is defined as the absence of a destructive immune response to a transplanted tissue in the setting of no immunosuppression [62]. Robust tolerance strategies involve the complete elimination of the need for immunosuppressive drugs and result in the recipient remaining fully capable of responding to other antigens. Graft-versus-host disease (GvHD) is a complication of allogeneic stem cell transplantation that causes significant morbidity and can be life-threatening. It involves the trafficking of donor T cells to specific areas of the body, namely the skin, intestine, and liver, where an immune response is then mounted against host cells [63]. Although chimerism-based tolerance induction has shown success in cases of living donor kidney transplantation,

the potential for developing GvHD remains a major concern, especially in the setting of stable donor stem cell engraftment [64–66]. The highest risk of GvHD is associated with human leukocyte antigen (HLA)-full-mismatched unrelated donors. Given the diversity of the HLA, with > 7000 alleles (encoding HLA-A, -B, -C, and DRB1) [67], full HLA mismatch is almost always the cause of GvHD in the setting of deceased tissue donation. Furthermore, VCA recipients face a continual threat of DSA development despite long-term immunosuppressive therapy efforts. The majority of DSAs are produced secondary to T cell-dependent B cell responses [20–22]. Therefore, the development of immune tolerance, which would permit the patient's immune system to downregulate alloreactive B cells and T cells, has the potential to prevent or significantly decrease the risk for AMR. Novel immunomodulatory approaches aimed at preventing GvHD and AMR are greatly needed to improve VCA patient outcomes.

8. Tolerance Induction Using CD3IT T Cell Depletion

Clinical VCA requires the use of deceased organ donors, which introduces unique logistical and immunological challenges for successful chimerism-based tolerance induction. Anti-cluster of differentiation 3 (CD3) immunotoxins (CD3ITs), which induce significant but transient in vivo T cell depletion by inhibiting protein synthesis in CD3+ cells, have been effective in large animal models of immune tolerance [68]. CD3IT has distinctly different properties from other lymphocyte-depleting agents currently used for transplantation induction therapy, such as Thymoglobulin® (ATG) and Alemtuzumab (Campath). While both ATG and CD3IT deplete T cells, their efficacy and specificity differ. ATG significantly reduces peripheral blood T lymphocytes; however, it is only moderately effective in lymph node T cell depletion. In contrast, CD3IT depletion occurs quickly, with a half-life of <2 h, and targets T cells in peripheral blood as well as in the lymphoid tissues. This suggests its potential as a more effective or complementary treatment in transplant protocols due to its targeted action and conservation of other immune cells [69]. This may benefit bone marrow engraftment as the depletion of T cells within the recipient bone marrow provides a competitive advantage to the donor stem cell graft.

Fuchimoto et al. were able to achieve stable long-term mixed chimerism following organ transplant in miniature swine using both a megadose of peripheral blood stem cells and CD3IT [70]. CD3IT has also been studied in the context of VCA in swine, which has led to the development of successful tolerance induction protocols for living donor transplantation that involve pre-treatment of the donor and recipient 4–5 days before transplantation [71,72]. In addition, the human anti-CD3 fusion protein A-dmDT390-bisFv(UCHT1) or VG712, (also known as Resimmune®) has been shown to be an effective in vivo T cell-depleting reagent in cutaneous T cell lymphoma patients [73]. These studies demonstrate that CD3IT induction facilitates stem cell engraftment without the need for myelosuppressive conditioning. Furthermore, CD3IT-mediated depletion differs among different T cell subpopulations depending on the density of CD3 expression on the cell surface. For example, effector T cells, which express more CD3 on their surface than regulatory T cells (Tregs) [74], experience a higher degree of depletion. Therefore, treatment with CD3IT results in transient depletion of T cells with a relative sparing of Tregs. This effect was observed by Kim et al. in recent studies involving a newly derived murine version of CD3IT with a similar depletion mechanism [75]. Additionally, recently published data suggest that ATG induction therapy may increase the potential for post-transplantation alloantibody responses [25], while studies using CD3IT induction in swine suggest CD3IT encourages an immune regulatory response to infused donor cells that may prevent DSA development and facilitate immune tolerance induction [76].

A study using CD3IT in non-human primates revealed that treatment resulted in a significant depletion of T follicular helper cells (Tfh) [77]. Tfh cells are fundamental for both the formation of germinal centers and the production of long-lived, high-affinity B cells [78]. Although the mechanisms of this finding are not fully elucidated, the ability of CD3IT to specifically target Tfh cells underscores its potential to reduce the generation of DSAs.

This, in turn, decreases the risk of AMR and improves long-term graft survival in VCA. It is important to note that the main side effect of IT therapies is vascular leak syndrome characterized by increased vascular permeability, causing leakage of fluids and proteins into the extracellular space [73]. It has been found that supportive care with albumin infusions and diuretics reduces the symptoms of this transient adverse side effect [73]. This suggests that excluding patients with preexisting heart and chronic liver diseases may diminish this complication. In summary, CD3IT sufficiently reduces alloreactive T cells while preserving long-term regulatory mechanisms that can be directed toward preventing infectious diseases and cancer rather than contributing to GvHD in VCA graft recipients. This novel tolerizing strategy should be strongly considered for the development of future VCA tolerizing protocols.

9. Conclusions

Considering the current inability to prevent the formation of DSAs and the significant adverse side effects of life-long immunotherapy, there is an obvious need for tolerizing strategies to decrease the risk of AMR and improve graft survival. Recent studies have demonstrated the development of DSAs in both preclinical animal VCA models and human facial and upper limb VCA grafts. Swine VCA research demonstrated a correlation between rejection patterns and anti-donor IgG antibody levels, while alloimmune responses significantly interfered with early engraftment and overall graft survival. The human VCA studies highlighted the importance of donor compatibility, early treatment of AMR-associated vasculopathy to prevent graft damage, and periodic monitoring of antibody levels. Current treatment plans for AMR include a combination of antibody depletion methods and agents that control the rejection process. However, the CD3IT-based tolerogenic approach offers an effective method of immunosuppression while preserving immune regulatory responses following VCA. Therefore, achieving a state of immune tolerance, through approaches such as CD3IT, would greatly reduce or eliminate the occurrence of AMR in VCA recipients. Overall, future efforts to achieve tolerance should be clinically safe with limited risk of toxicity and graft function impairment.

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